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Indole-3-carbinol prevents diet-induced obesity through modulation of multiple genes related to adipogenesis, thermogenesis or inflammation in the visceral adipose tissue of mice☆

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Abstract

Indole-3-carbinol (I3C) is a compound found in high concentrations in *Brassica* family vegetables, including broccoli, cauliflower and cabbage, and is regarded as a promising chemopreventive agent against various cancers. This study assesses the protective effect of I3C against diet-induced obesity in mice. Mice were randomly grouped to receive either a normal diet, high-fat (40% energy as fat) diet (HFD) or I3C-supplemented diet (1 g/kg diet) for 10 weeks. I3C supplementation significantly ameliorated HFD-induced increases in body weight gain, visceral fat pad weights and plasma lipid levels. The visceral adipose tissue mRNA levels of uncoupling proteins 1 and 3, crucial factors of thermogenesis, and their regulators such as sirtuin 1, peroxisome proliferator-activated receptor (PPAR) α and PPARγ coactivator 1α, which were down-regulated by HFD, were normalized by supplementation with I3C. In contrast, I3C supplementation significantly decreased expression levels of a key adipogenic transcription factor, PPARγ2, and its target genes, such as leptin and adipocyte protein 2, in the visceral adipose tissue of mice maintained on the HFD. Furthermore, HFD-induced up-regulation in mRNA levels of inflammatory cytokines (tumor necrosis factor α, interferon β and interleukin 6) was significantly ameliorated by I3C. These findings suggest that I3C has a potential benefit in preventing obesity and metabolic disorders, and the action for I3C *in vivo* may involve multiple mechanisms including decreased adipogenesis and inflammation, along with activated thermogenesis.

Keywords: Antiobesity; Indole-3-carbinol; Adipogenesis; Inflammation; Thermogenesis

1. Introduction

The adipose tissue is an endocrine organ which plays a fundamental role in metabolism and homeostasis regulation through the secretion of several biologically active adipokines including cytokines and chemoattractant proteins along with molecules involved in glucose and lipid metabolism, adipogenesis and thermogenesis [1]. For this reason, obesity, especially increased visceral adiposity, leads to increased production of several inflammatory cytokines that play a critical role in obesity-related inflammation and metabolic pathologies [2]. Tumor necrosis factor-alpha (TNFα) is a potent cytokine that induces the production of interleukin-6 (IL-6), which is the major determinant of the acute phase response, and also has effects on glucose transport, lipid metabolism and insulin action [2]. It has been reported that, in obese individuals and animal models, the levels of TNFα and IL-6 are persistently elevated, whereas a reduction of adipose tissue mass leads to a decrease in these expression levels [2]. There is increasing evidence for the fundamental role of toll-like receptors (TLRs) in linking nutrition, adipose tissue and innate immunity. TLRs play an important role in regulating innate immune responses to both infectious and sterile inflammatory stimuli, and free fatty acids (FFAs) are important ligands for TLR2 and TLR4. TLRs

Abbreviations: aP2, adipocyte protein 2; CD36, cluster of differentiation 36; CTSS, cysteine protease cathepsin S; DIM, diindolylmethane; FFAs, free fatty acids; ERK, extracellular signal-regulated kinase 1/2; GalR1, galanin receptor 1; HFD, high-fat diet; IFNβ, interferon-beta; IL-6, interleukin 6; I3C, Indole-3-carbinol; IRF5, interferon regulatory factor 5; MyD88, myeloid differentiation primary response gene 88; ND, normal diet; PGC1α, PPARγ coactivator 1α; PKCδ, protein kinase C delta; PPARγ2, peroxisome proliferator-activated receptor gamma 2; SIRT1, sirtuin 1; Tirap, toll-interleukin 1 receptor (TIR) domain-containing adaptor protein; TLR2, toll like receptor 2; TLR4, toll like receptor 4; TNFα, tumor necrosis factor-alpha; TRAF6, TNF receptor-associated factor 6; TRIF, TIR-domain-containing adapter-inducing interferon-β; UCP, uncoupling protein.

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therefore represent a direct molecular link between hyperlipidemia, a central clinical feature of obesity, and activation of the innate immune system. TLR2 and TLR4 are expressed in a wide range of cells, including macrophages and adipocytes, and upon binding FFAs, activate NFκB, upregulate inflammatory cytokine expression and induce insulin resistance [3].

Adipose tissue growth, such as that observed during normal development and obesity, is the result of both hypertrophy (increase in size) and hyperplasia (increase in number) of adipocyte [4]. Adipogenesis is mainly regulated by peroxisome proliferator activated receptor gamma (PPARγ), a member of the nuclear receptor family that serves as the master transcriptional regulator. Downstream targets for PPARγ include a host of genes involved in lipid accumulation and metabolism, such as adipocyte lipid binding protein (aP2), cluster of differentiation 36 (CD36), lipoprotein lipase and phosphoenolpyruvate carboxykinase [5]. Increased expression and/or activation of uncoupling proteins (UCPs) in the adipose tissue uncouples oxidative phosphorylation, resulting in the conversion of energy to heat [6]. Therefore, it is possible that drugs activating or increasing the expression of UCP1 and UCP3 would have important effects on energy expenditure or on the rate of nutrient oxidation.

Indole-3-carbinol (I3C, 1H-indol-3-methanol, C_9H_9NO) is a naturally occurring compound found in cruciferous vegetables of the genus *Brassica*, which includes cabbage, broccoli, cauliflower, brussel sprouts, turnip, kale and kohlrabi. I3C is produced when these vegetables are macerated, cut or cooked *via* the myrosinase-catalyzed hydrolysis of glucobrassicin, an indole glucosinolate [7]. Anderton et al. reported that after oral administration of I3C (250 mg/kg) to mice, I3C was rapidly absorbed and had already reached an apparent peak concentration of 4.1 μg/ml at the earliest sampling time point of 15 min in plasma. Furthermore, maximal plasma I3C concentrations observed were considerably higher than those for its acid condensation products such as diindolylmethane (DIM) (I3C, 4.1 μg/ml vs. DIM, 0.2 μg/ml) [8]. At the concentration range of 50–100 μM, I3C has been shown to suppress the proliferation of various cancer cells, including those of breast [9], colon [10], prostate [11] and endometrium [12] cancers, by targeting a wide spectrum of signaling pathways governing hormonal homeostasis, cell-cycle progression, and cell proliferation and survival [13]. Although I3C has been shown to possess anticancer properties, its antiobesity effect has not been studied yet. The main objective of this study was to investigate the weight-lowering effect of I3C supplementation in mice maintained on the high-fat diet (HFD) and to study the potential molecular mechanisms of this effect, focusing on the expression of genes involved in adipogenesis, thermogenesis, and inflammation in the adipose tissue.

2. Materials and methods

2.1. Materials

3T3-L1 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). I3C (MW 147.17 g/mol, 96% purity) and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Bovine calf serum (BCS) was purchased from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS), penicillin/streptomycin and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Hyclone (Logan, UT, USA). Isopropanol was purchased from Amresco (Solon, OH, USA). Antibodies for MAP kinase [extracellular signal-regulated kinase 1/2 (ERK)], phospho-ERK (Thr202/Tyr204), IRF3 and phospho-IRF3 (Ser396) were purchased from Cell Signaling Technology (Beverly, MA, USA), and the antibody against β-actin was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Cell culture and oil red O staining

3T3-L1 cells were cultured at 37°C in a humidified 5% CO_2 atmosphere and grown in a culture medium (DMEM supplemented with 10% BCS and 1% penicillin/streptomycin). After the 3T3-L1 cells became confluent, the medium was replaced with differentiation medium containing 10% FBS, 1% penicillin/streptomycin, 1 μg/ml insulin, 1 μM dexamethasone and 0.5 mM isobutylmethylxanthine and cultured for 2 days. The cell medium was then replaced with medium containing only 10% FBS plus 1 μg/ml insulin, and the medium was replaced every 2 days. The cells were treated with varying doses of I3C (0.1, 1, 10, 50 and 100 μM) for 10 days during differentiation. On day 10, cells were washed twice with phosphate-buffered saline, fixed with 10% formalin for 1 h at room temperature and then stained with 5% oil red O in isopropanol for 30 min. Cells were then washed with water, and images of each dish were taken using Olympus (Tokyo, Japan) microscope. Stained oil droplets were dissolved in isopropanol and quantified by spectrophotometrical analysis at 600 nm.

2.3. Animal care and experimental protocol

All mice were housed in the specific pathogen-free facility of the Yonsei University, Seoul, Korea, and this study was approved by the Institutional Animal Care and Use Committee of Yonsei University. Twenty-four male C57BL/6N mice (5 weeks old) purchased from Orient Bio (Gyeonggi-do, South Korea) were housed in a pathogen-free facility with 21°C±2.0°C temperature, 50%±5% relative humidity and a 12-h light/12-h dark cycle. All of the mice consumed a commercial diet and tap water *ad libitum* for 1 week prior to their division into three weight-matched groups (n=8 per group): normal diet (ND), HFD and I3C-supplemented diet (I3CD) groups. The ND was a purified diet based on the AIN-76 rodent diet composition. The HFD was identical to the ND, except that 200 g fat/kg (170 g lard plus 30 g corn oil) and 1% cholesterol were added to it. The HFD (20% fat, wt/wt) was formulated to provide 40% of the total energy generated by the diet from fat by replacing carbohydrates with lard and corn oil, but it contained the same amount of vitamins and minerals per kilojoule as the ND did. The I3CD was identical to HFD and contained 0.1% (wt/wt) I3C. At the end of the experimental period, the animals were anesthetized with ether following a 16-h fast. Blood was drawn from the abdominal aorta into an EDTA-coated tube, and the plasma was obtained by centrifuging the blood at 2000*g* for 15 min at 4°C. The epididymal, retroperitoneal, mesenteric and perirenal fat pads were dissected out, weighed and snap frozen in liquid nitrogen prior to their storage at −80°C.

2.4. Biochemical analysis

Plasma concentrations of total cholesterol, triglyceride (TG), FFA and glucose were enzymatically determined using commercial kits (Bio-Clinical System, Gyeonggi-do, Korea). Plasma insulin levels were measured through radioimmunoassay (Linco Research, Inc., St. Louis, MO, USA). The homeostasis model assessment of basal insulin resistance (HOMA-IR) was used to calculate an index from the product of the fasting concentrations of plasma glucose (mmol/L) and insulin (pmol/L) divided by 22.5. Lower HOMA-IR values indicated greater insulin sensitivity, whereas higher HOMA-IR values indicated lower insulin sensitivity (insulin resistance). Plasma levels of TNFα and monocyte chemoattractant protein-1 (MCP1) were measured using enzyme-linked immunosorbent assay kits (ID Labs, MA, USA).

2.5. RNA extraction and semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR)

The total RNA was isolated from the epididymal and retroperitoneal adipose tissues of each mouse using Trizol (Invitrogen, CA, USA) and was reverse-transcribed using the Superscript II kit (Invitrogen, CA, USA) according to the manufacturer's recommendations. The forward (F) and reverse (R) primers for each mouse gene are shown in Table 1. The PCR was programmed as follows: 10 min at 94°C, 30–35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min and 10-min incubation at 72°C.

2.6. Western blot analysis

The epididymal adipose tissues of each mouse were homogenized at 4°C in an extraction buffer containing 100 mM Tris-HCl, pH 7.4, 5 mM EDTA, 50 mM NaCl, 50 mM sodium pyrophosphate, 50 mM NaF, 100 mM orthovanadate, 1% Triton X-100, 1 mM phenylmethanesulphonyl fluoride, 2 μg/ml aprotinin, 1 μg/ml pepstatin A and 1 μg/ml leupeptin. The tissue homogenates were centrifuged (1300*g*, 20 min, 4°C), and the resulting supernatants (whole-tissue extracts) were used for the Western blot analysis. The total protein concentrations of whole-tissue extracts were determined via Bradford assay (Bio-Rad, CA, USA). The protein samples were separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis and were transferred onto the nitrocellulose membrane (Amersham, Buckinghamshire, UK) and hybridized with primary antibodies (diluted 1:1000) overnight at 4°C. After the membrane was again incubated with the relative secondary antibody, immunoreactive signals were detected using the chemiluminescent detection system (Amersham, Buckinghamshire, UK) and were quantified using the Quantity One analysis software (Bio-Rad, CA, USA).

2.7. Statistical analysis

Results were expressed as the mean±S.E.M. of values for eight animals. Comparisons among groups were made using one-way analysis of variance. The differences between the mean values in the three groups were tested through Duncan's multiple-range test and were considered significant when the P value was less than .05.

3. Results

3.1. I3C inhibits 3T3-L1 cell differentiation

I3C decreased the cell population growth in a dose-dependent manner with an IC_{50} value of 35.2 mM against the 3T3-L1 preadipocytes (data not shown). To study the potential inhibitory effects of I3C on adipogenesis, various concentrations of I3C were added to the differentiation medium for 10 days during the culture of 3T3-L1 cells. Macroscopic observations of oil red O staining are shown in Fig. 1A. With increasing concentrations of I3C in culture media, a significant inhibition of differentiation of preadipocytes to mature adipocytes was observed, and it was evident from reduced fat accumulation in the cells. I3C at 10-, 50- and 100-μM concentrations decreased cellular lipid content by 20%, 53% and 62%, respectively, when compared with cells not treated with I3C (Fig. 1B). These results indicate that I3C may have efficiently blocked adipocyte differentiation in a cultured 3T3-L1 cell line. The effects of I3C on mRNA expressions of adipogenic genes during 3T3-L1 adipocyte differentiation are shown in Fig. 1C. The mRNA expressions of C/EBPα (−67%), PPAR γ2 (−48%), and aP2 (−45%) by 3T3-L1 adipocytes were significantly reduced by I3C (100 μM).

3.2. I3C decreases body and visceral fat pad weights and plasma lipid levels

I3C supplemented to mice fed an HFD significantly reduced the final body weight (−36%) and body weight gain (−59%) (Fig. 2A, B) without affecting food intake (Fig. 2C). The food efficiency ratio of the I3CD-fed mice was 55% lower than that of the HFD-fed mice ($P<.05$) (Fig. 2D). I3C supplementation significantly reduced total visceral fat pad weight by 49% in mice fed the HFD, and this was attributed to weight decreases in the epididymal (−44%, $P<.05$), retroperitoneal (−46%, $P<.05$), mesenteric (−57%, $P<.05$) and perirenal (−74%, $P<.05$) fat depots (Fig. 2E, F). I3C significantly attenuated the HFD-induced elevations in the plasma TG (by 72%), FFA (by 83%), total cholesterol (by 53%), TNFα (−45%) and MCP1 (−58%) levels (Table 2). I3C significantly reversed the HFD-induced elevations in plasma glucose (−34%) and insulin (−47%) levels. The insulin sensitivity was increased significantly by I3C supplementation as indicated by the 65% reduction in HOMA-IR (Table 2).

3.3. I3C modulates the expression of molecules involved in adipogenesis or thermogenesis

Mice fed the I3CD exhibited significant reductions in the mRNA levels of galanin receptor 1 (GalR1, −62%), cysteine protease cathepsin S (CTSS, −49%) and the key adipogenic transcription factor PPARγ2 (−29%) along with its target genes, such as aP2 (−45%) and leptin (−19%), in their epididymal adipose tissues compared with the HFD-fed mice (Fig. 3A). The results from the immunoblots of proteins isolated from the epididymal adipose tissue confirmed that ERK phosphorylation was significantly down-regulated in the I3CD-fed mice (−42%) compared with the HFD-fed mice (Fig. 3B). Compared

Table 1
Primer sequences used for RT-PCR

Gene description	Primers	Sequences (5′→3′)	Annealing temperature (°C)	PCR product (bp)
GalR)	F	CCAAGGGGGTATCCCAGTAA	60	134
	R	GGCCAAACACTACCAGCGTA		
CTSS	F	TCCCGGTCTTTGGCTATTTT	55	149
	R	CCTCCACAGCCTTTATTCCC		
PPARγ2	F	TTCGGAATCAGCTCTGTGGA	55	148
	R	CCATTGGGTCAGCTCTTGTG		
aP2	F	AGCATCATAACCCTAGATGG	55	128
	R	GAAGTCACGCCTTTCATAAC		
Leptin	F	CTCCAAGGTTGTCCAGGGTT	55	143
	R	AAAACTCCCCACAGAATGGG		
SIRT1	F	AGCTCCTTGGAGACTGCGAT	55	182
	R	ATGAAGAGGTGTTGGTGGCA		
PGC1α	F	ACTGACAGATGGAGCCGTGA	55	180
	R	GCTGCATGGTTCTGAGTGCT		
PPARα	F	GAACCCAAGTTTGACTTCGCT	55	136
	R	CCTATGTTTAGAAGGCCAGGC		
UCP1	F	CTGGGCTTAACGGGTCCTC	55	100
	R	CTGGGCTAGGTAGTGCCAGTG		
UCP3	F	ATGCTGAAGATGGTGGCTCA	55	179
	R	TTGCCTTGTTCAAAACGGAG		
TLR2	F	GAGCATCCGAATTGCATCAC	55	120
	R	TATGGCCACCAAGATCCAGA		
TLR4	F	TCGAATCCTGAGCAAACAGC	55	199
	R	CCCGGTAAGGTCCATGCTAT		
Tirap	F	GCTTCCAGGGGATCTGATGT	55	183
	R	AAGCAAGCCTACCACGGACTAAG		
MyD88	F	AAAGTGAGTCTCCCCTC	55	149
	R	TCCCATGAAACCTCTAACAC		
TRAF6	F	GCACAAGTGCCCAGTTGACAATGA	55	689
	R	AGTGTCGTGCCAAGTGATTCCTCT		
IRF5	F	ACCCGGATCTCAAAGACCAC	55	166
	R	TTATTGCATGCCAACTGGGT		
TRIF	F	ATGGATAACCCAGGGCCTT	55	528
	R	TTCTGGTCACTGCAGGGGAT		
TNFα	F	TGTCTCAGCCTCTTCTCATT	55	156
	R	AGATGATCTGAGTGTGAGGG		
IFNβ	F	TGGAGCAGCTGAATGGAAAG	55	122
	R	GAGCATCTCTTGGATGGCAA		
IL-6	F	ATGAAGTTCCTCTCTGCAAGAGA	55	638
	R	CACTAGGTTTGCCGAGTAGATCTC		
Glyceraldehyde-3-phosphate dehydrogenase	F	AGAACATCATCCCTGCATCC	55	321
	R	TCCACCACCCTGTTGCTGTA		

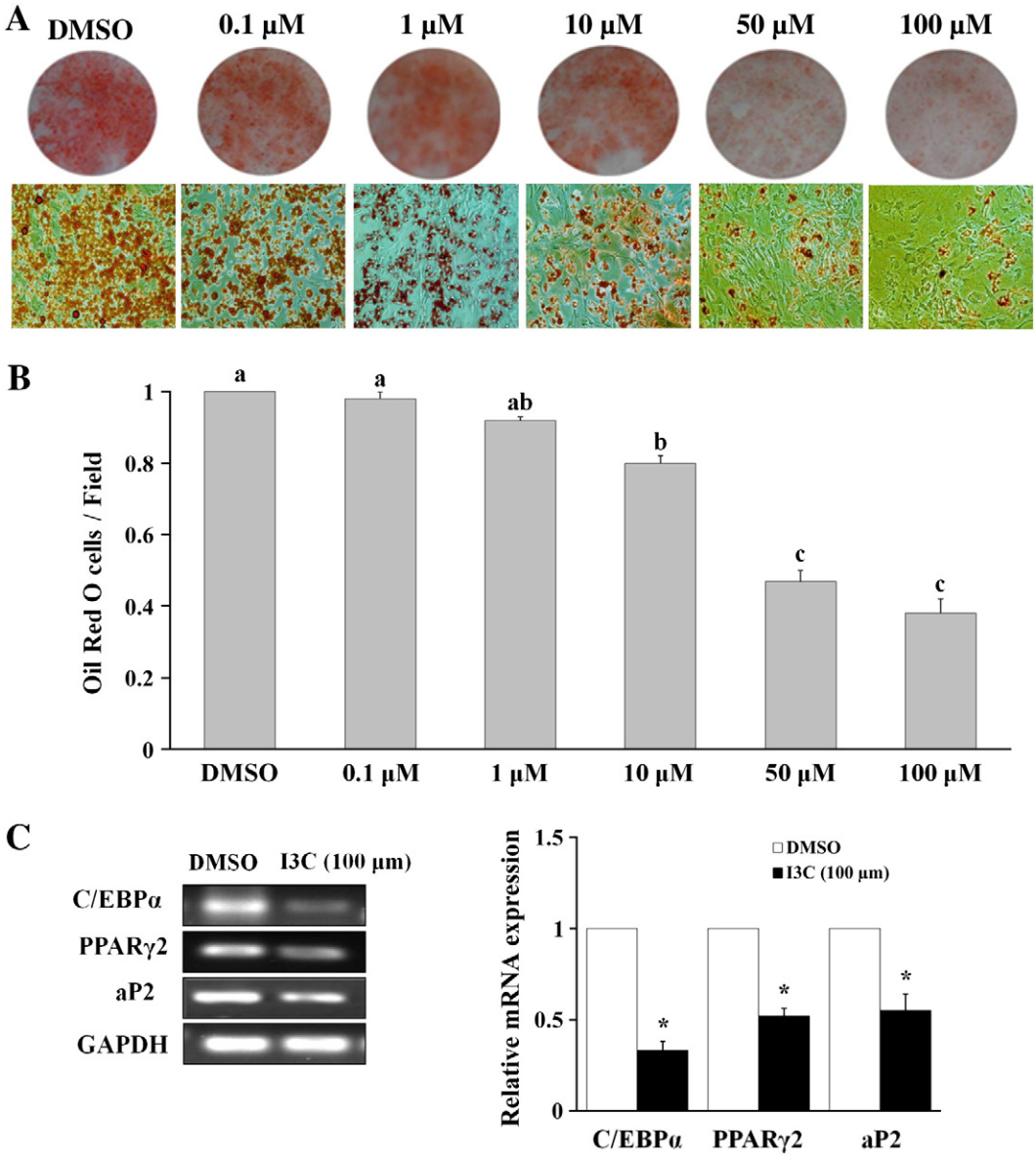

Fig. 1. Effect of I3C on 3T3-L1 adipocyte differentiations. Postconfluent 3T3-L1 cells were incubated for 48 h during the adipogenic induction in culture medium and treated with the indicated concentrations of I3C. (A) Ten days after the onset of induction, adipocytes were fixed, stained with oil red O and examined both macroscopically and microscopically. Magnification, ×200. (B) Cells were harvested, and the lipid accumulation was measured through a spectrophotometer. (C) Effect of I3C on mRNA expressions of adipogenic genes during 3T3-L1 adipocyte differentiation. These values shown represent the mean±S.E.M. of triplicate determinations from three independent experiments. Means not designated by a common superscript are different, $^{abc}P<.05$.

with the ND group, the HFD mice showed significantly decreased mRNA levels of genes related to thermogenesis, such as sirtuin 1 (SIRT1), PPARγ coactivator 1α (PGC1α), PPARα, UCP1 and UCP3, in their epididymal adipose tissues. Feeding the mice with I3CD significantly restored the HFD-induced down-regulations of SIRT1 (a 40% increase), PGC1α (a 64% increase), PPARα (a 46% increase), UCP1 (a 72% increase) and UCP3 (a 46% increase) in their epididymal adipose tissues (Fig. 3C). Furthermore, the mRNA expressions of UCP1 (a 61% increase) and UCP3 (a 38% increase) were also higher in the brown adipose tissue of mice fed an I3C-supplemented diet than those of mice fed an HFD (Fig. 3D).

3.4. I3C modulates the expression of molecules involved in inflammation

We next examined the anti-inflammatory properties of I3C in the epididymal adipose tissue of mice maintained on the HFD. I3C significantly down-regulated the mRNA expressions of TLR2 and TLR4 compared to the levels in the HFD-fed mice. Animals fed the I3CD exhibited significantly down-regulated mRNA levels of toll-interleukin 1 receptor domain-containing adaptor protein (Tirap), myeloid differentiation primary response gene 88 (MyD88), TNF receptor-associated factor 6 (TRAF6), interferon regulatory factor 5 (IRF5) and TIR-domain-containing adapter-inducing interferon-β (TRIF) in their epididymal adipose tissues compared to the HFD-fed mice. Moreover, the mRNA levels of proinflammatory cytokines, including TNFα, interferon-beta (IFNβ) and IL-6, were also significantly lower in the I3CD-fed mice than in the HFD-fed mice (Fig. 4A). The immunoblot results confirmed that IRF3 phosphorylation was significantly down-regulated in the epididymal adipose tissue of I3CD-fed mice (−76%) compared with phosphorylation in tissue from the HFD-fed mice (Fig. 4B).

To determine whether the I3C has direct anti-inflammatory effects on adipocyte, we examined the mRNA expressions of TLR pathway-

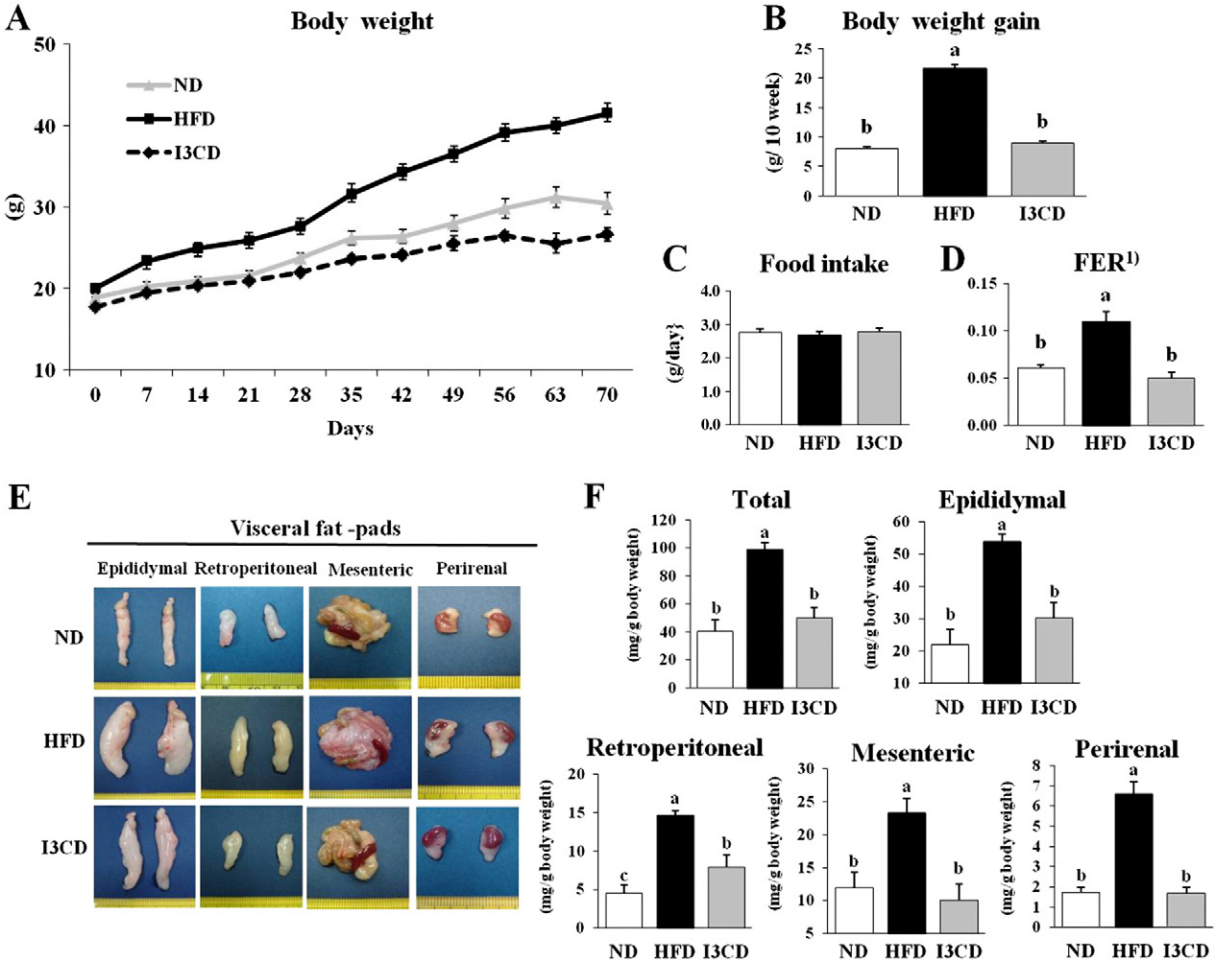

Fig. 2. Effect of I3C on (A) body weight changes, (B) body weight gain, (C) food intake, (D) food efficiency ratio (FER) and (E, F) visceral fat pad weights in HFD-fed mice. The values are the means±S.E.M. of eight mice. Means not designated by a common superscript are different, $^{abc}P<.05$.

associated genes in 3T3-L1 adipocyte in which inflammation is elicited by FFAs. The results showed that, in 3T3-L1 adipocytes, palmitic acid (0.5 mmol/L, 24 h) induced the mRNA expressions of TLR2, TLR4, MyD88, TRAF6, TRIF and proinflammatory cytokines. However, I3C significantly reversed the FFA-induced up-regulations of TLR2 (−92%), TLR4 (−90%), MyD88 (−46%), TRAF6 (−56%), TRIF (−158%) and proinflammatory cytokines, such as TNFα (−769%), IFNβ (−790%) and IL-6 (−216%), in 3T3-L1 adipocytes (Fig. 4C).

4. Discussion

The effect of I3C supplementation on body weight loss did not depend on decreased food or energy intake because there were no significant differences in food intake among groups. In the present study, I3C supplemented to the HFD significantly suppressed the expressions of GalR and CTSS in the epididymal adipose tissue of mice. Galanin is one of the most inducible neuropeptides that is known to stimulate the intake of a fat-rich diet [14]. The conventional galanin-mediated signaling involves the activation of the Ras/c-Raf/mitogen-activated protein kinase kinase/ERK (*via* GalR1) and protein kinase C-δ (PKC-δ)/ERK (*via* GalR2) pathways, which have been reported in the nerve systems and tumor cells [15,16]. A recent report suggested that the activation of the galanin-mediated signaling pathways appears to play a role, at least in part, in the development of HFD-induced adipogenesis in the visceral adipose tissue of mice because the prolonged consumption of an HFD led to increases in the expression of the galanin-mediated signaling molecules, such as GalR1and 2, Ras, c-Raf, PKC-δ and ERK, in the peripheral tissues [17]. Meanwhile, CTSS, a potent cysteine protease known to be involved in atherogenesis, angiogenesis and inflammation processes [18], has been identified as a novel adipose tissue biomarker that is over-expressed in obesity [19]. The role of CTSS in human preadipocytes was highlighted by the finding that the inhibition of its activity leads to decreased adipogenesis, whereas supplementation of culture media with CTSS promotes adipogenesis [20].

In the present study, I3C supplementation reversed the HFD-induced down-regulations of SIRT1, PGC1α, UCP1 and UCP3 expression, all of which are crucial factors of thermogenesis, in the visceral

Table 2
Plasma biochemistries of mice fed experimental diets

Groups	ND	HFD	I3CD
Plasma			
TG (mmol/L)	0.60 ± 0.09^{b}	1.41 ± 0.12^{a}	0.39 ± 0.09^{b}
FFA (mEq/L)	573 ± 36.3^{b}	1416 ± 128^{a}	236 ± 46.9^{c}
Total cholesterol (mmol/L)	2.02 ± 0.06^{b}	3.83 ± 0.32^{a}	1.82 ± 0.11^{b}
Glucose (mmol/L)	5.42 ± 1.07^{b}	10.27 ± 1.03^{a}	6.73 ± 0.92^{b}
Insulin (pmol/L)	103 ± 4.31^{b}	191 ± 6.35^{a}	101 ± 5.65^{b}
HOMA-IR	24.8 ± 0.92^{b}	87.1 ± 3.26^{a}	30.2 ± 2.97^{b}
TNFα (pg/ml)	36.9 ± 3.66^{c}	107.4 ± 4.69^{a}	58.9 ± 4.32^{b}
MCP1 (pg/ml)	15.1 ± 2.23^{c}	81.8 ± 4.31^{a}	34.1 ± 5.11^{b}

Values are expressed as mean±S.E.M. ($n=8$). Values within a row without a common letter differ significantly, $P<.05$.

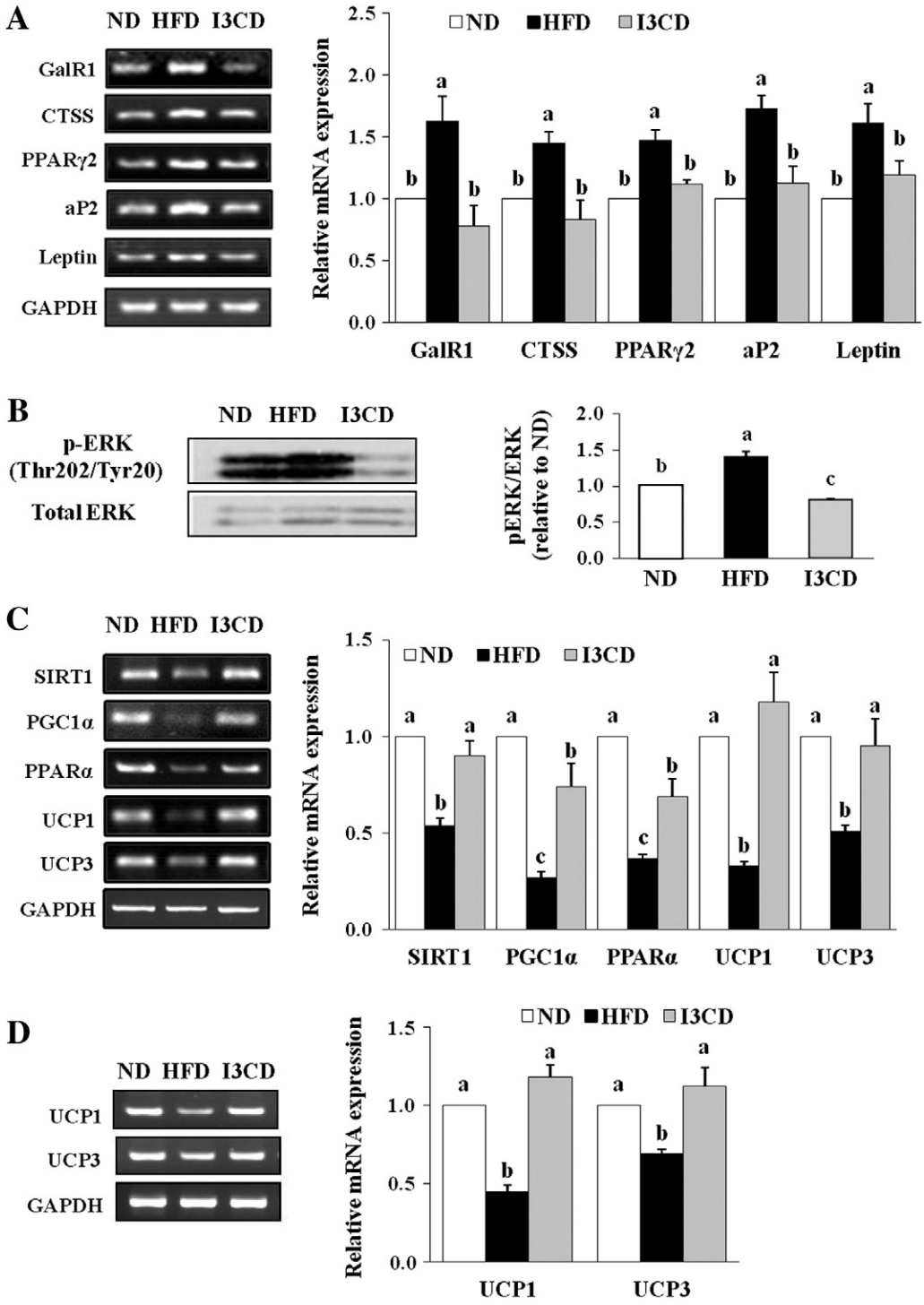

Fig. 3. Effect of I3C on the regulation of adipose tissue genes implicated in adipogenesis or thermogenesis in mice maintained on the HFD. (A) Semiquantitative RT-PCR and (B) Western blot analyses of adipogenic molecules in the epididymal adipose tissues. (C) Semiquantitative RT-PCR of genes related to thermogenesis in the epididymal adipose tissues. (D) Semiquantitative RT-PCR of UCP1 and UCP3 in the brown adipose tissues. The RT-PCR and immunoblot data were presented as the means±S.E.M. of eight mice. Means not designated by a common superscript are different, $^{abc}P<.05$. 1)FER(food efficiency ratio) $= \frac{\text{Body weight gain for experimental period (g)}}{\text{Food intake for the experimental period (g)}}$.

adipose tissue of mice. Adaptive thermogenesis is defined operationally as heat production in response to environmental temperature or diet, and serves the purpose of protecting the organism from cold exposure or regulating energy balance after changes in diet [21]. It now seems evident that SIRT1 is a key molecule that promotes thermogenesis at least partly through its effect on metabolic homeostasis. SIRT1 physically interacts with and deacetylates PGC1α at multiple lysine sites, consequently increasing PGC1α activity and leading to the induction of UCP gene transcription [22]. PPARs (α/β/γ2) can activate the UCP1 and UCP3 enhancer in preadipocytes, indicating that molecules interacting with PPARs could represent key components of the thermogenic response in

adipose tissue. In addition to PPARs (α/β/γ2), PGC1α also binds to a variety of other nuclear receptors including retinoic acid and thyroid hormone receptors, both of which positively regulate the expression of UCP1 and UCP3, which are involved in rodent temperature and body-weight regulation [21]. Increased expressions of these proteins uncouple oxidative phosphorylation, resulting in increased energy expenditure and decreased fat accumulation in rodents [23]. Recently, SIRT1 has been shown to function together with PGC1α to increase fat oxidation in a rodent model with diet-induced obesity [22]. Moreover, PPARα plays an essential role in maintaining lipid homeostasis by modulating both constitutive and inducible expression of genes that regulate fat oxidation [24].

It now appears that obesity is associated with a low-grade inflammation of white adipose tissue due to chronic activation of the innate immune system [25], and the "sensors" that potentially link obesity to inflammation are the toll-like family of receptors (TLRs) [26]. Activation of TLRs results in the synthesis of proinflammatory factors such as TNFα, IL-6 and chemokines [27]. Considering the implication of increased circulatory nonesterified fatty acid in adipose tissue dysfunction and of FFAs being direct ligands for TLR2 and 4 [28], it is very likely that the activation of TLRs takes place in hyperlipidemic states, resulting in amplified inflammation and contributing to the development or aggravation of the metabolic disorders [29]. TLR2 and TLR4, when stimulated with

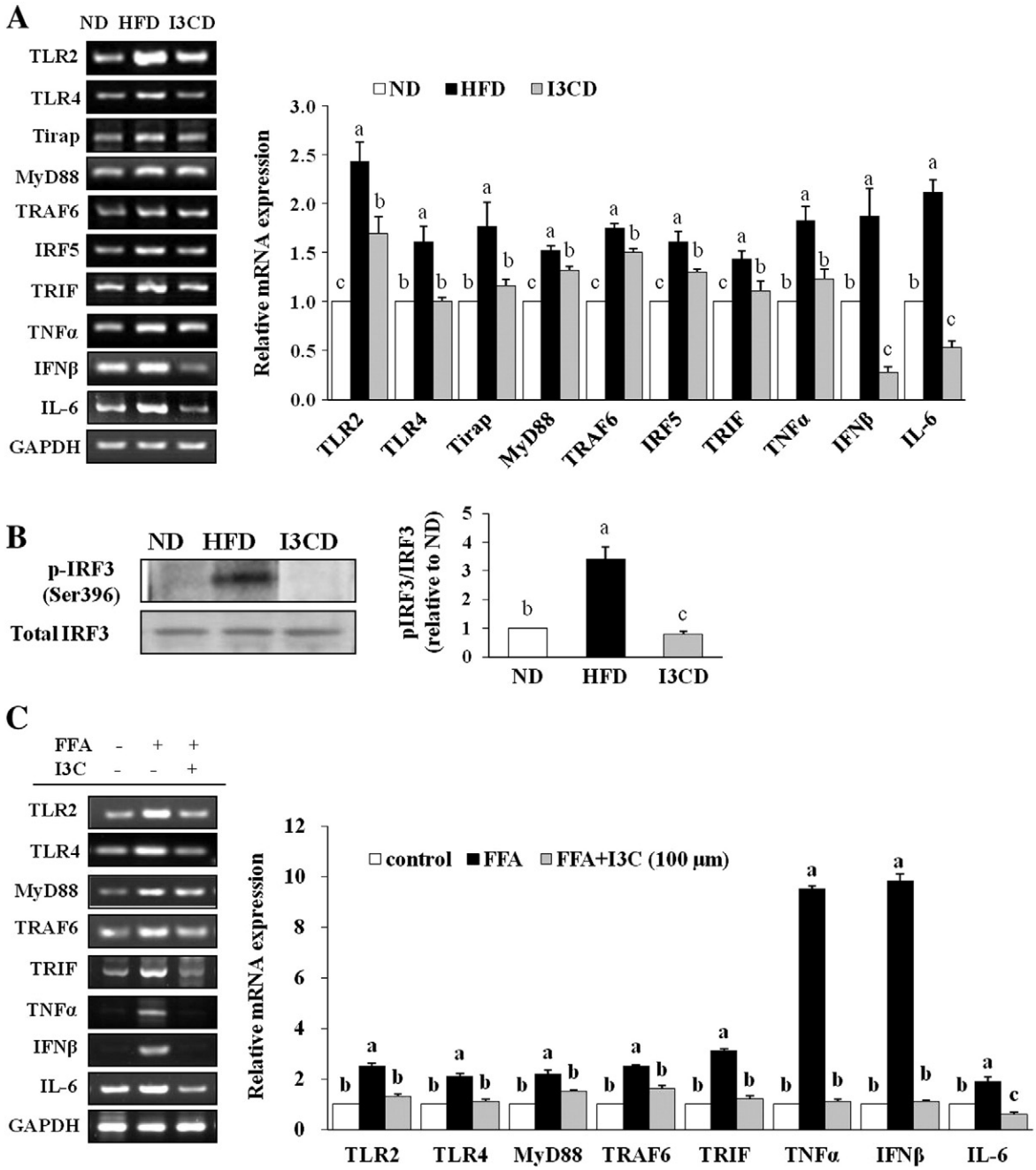

Fig. 4. (A) Semiquantitative RT-PCR analysis and (B) Western blot analyses of TLR-mediated proinflammatory signaling molecules in the epididymal adipose tissue of mice. (C) Semiquantitative RT-PCR analysis of TLR-mediated proinflammatory signaling molecules in 3T3-L1 adipocytes. The RT-PCR and immunoblot data were presented as the means±S.E.M of eight mice. Means not designated by a common superscript are different, $^{abc}P<.05$.

agonists, recruit adaptor molecules (MyD88, Tirap, TRIF and TRAF6) to activate signaling cascades that control the activities of numerous downstream transcription factors, including NFkB, AP-1 and IRF family members [30]. The mRNA and protein phosphorylation levels of TLR2 and TLR4 along with their downstream molecules (MyD88, Tirap, TRIF, TRAF6, IRF5 and IRF3) and target cytokines (TNFα, IFNβ and IL-6) were significantly reduced in the visceral adipose tissues of the I3CD-fed mice compared to the HFD mice. Based on these results, we speculate that I3C may attenuate obesity-induced inflammation probably through the suppression of TLR2- and TLR4-mediated proinflammatory signaling cascades.

In conclusion, our findings provide a biochemical and molecular basis for the use of I3C as a dietary bioactive compound, which may have important implications for preventing visceral adiposity and hyperlipidemia. The action for I3C *in vivo* may involve multiple mechanisms including decreased adipogenesis and inflammation along with activated thermogenesis in the visceral adipose tissue.

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